

IN THE CLAIMS:

Specific Instructions for Claim Amendments:

Please cancel Claims 1-31, without prejudice to or disclaimer of the subject matter therein.

Please add new Claims 32-85, as shown below.

Listing of Claims:

1-31. (Cancelled)

32. (New) A method for obtaining a soluble protein having at least one added free cysteine residue comprising the steps of:

a) obtaining an isolated host cell capable of expressing a soluble protein, wherein the soluble protein contains at least one added free cysteine;

b) exposing the host cell to a cysteine blocking agent prior to step (c), wherein the cysteine blocking agent forms a stable, mixed disulfide with at least one cysteine residue in the soluble protein; and

c) isolating the soluble protein from the host cell.

2 33. (New) The method of claim 32, wherein the step (b) of exposing comprises disrupting the host cell in the presence of the cysteine blocking agent, and wherein the step (c) of isolating comprises isolating the soluble protein containing a free cysteine residue from the soluble fraction of the disrupted host cell.

3 34. (New) The method of claim 32, wherein step (b) of exposing the host cell to a cysteine blocking agent occurs before, during or after synthesis of the soluble protein containing a free cysteine residue by the host cell and wherein the soluble protein containing a free cysteine residue is secreted from the host cell.

4 35. (New) A method for obtaining a soluble protein having at least one added free cysteine residue comprising the steps of:

a) obtaining an isolated host cell capable of expressing a soluble protein, wherein the soluble protein contains at least one added free cysteine;

b) exposing the host cell to a cysteine blocking agent selected from the group consisting of cysteine, ^{or a derivative thereof,} cystamine, dithioglycolic acid or oxidized glutathione,

~~or a derivative thereof~~, prior to step (c), wherein the cysteine blocking agent forms a stable, mixed disulfide with at least one cysteine residue in the soluble protein; and

c) isolating the soluble protein from the host cell.

5 36. (New) The method of claim 35, wherein the step (b) of exposing comprises disrupting the host cell in the presence of the cysteine blocking agent, and wherein the step (c) of isolating comprises isolating the soluble protein containing a free cysteine residue from the soluble fraction of the disrupted host cell.

6 37. (New) The method of claim 35, wherein step (b) of exposing the host cell to a cysteine blocking agent occurs before, during or after synthesis of the soluble protein containing a free cysteine residue by the host cell and wherein the soluble protein containing a free cysteine residue is secreted from the host cell.

7 38. (New) A method for obtaining a soluble protein having at least one added free cysteine residue comprising the steps of:

a) obtaining an isolated host cell ~~capable of~~ expressing and secreting a soluble protein into the media, wherein the soluble protein contains at least one added free cysteine;

b) exposing the media containing the soluble protein to a cysteine blocking agent prior to step (c), wherein the cysteine blocking agent forms a stable, mixed disulfide with at least one cysteine residue in the soluble protein; and

c) isolating the soluble protein from the media.

8 39. (New) The method of claim 38, wherein the cysteine blocking agent is added to the media before, during or after synthesis of the soluble protein containing a free cysteine residue by the host cell.

9 40. (New) A method for obtaining a soluble protein having at least one added free cysteine residue comprising the steps of:

a) obtaining an isolated host cell ~~capable of~~ expressing and secreting a soluble protein into the media, wherein the soluble protein contains at least one added free cysteine;

b) exposing the media containing the soluble protein to a cysteine blocking agent selected from the group consisting of cystine, ^{or a derivative hereof,} cystamine, dithioglycolic acid or oxidized glutathione, ~~or a derivative thereof~~, prior to step (c), wherein the cysteine blocking agent forms a stable, mixed disulfide with at least one cysteine residue in the soluble protein; and

c) isolating the soluble protein from the media.

¹⁰~~41~~. (New) The method of claim ⁹~~40~~, wherein the cysteine blocking agent is added to the media before, during or after synthesis of the soluble protein containing a free cysteine residue by the host cell.

¹¹~~42~~. (New) The method of any one of claims ^{1 4 7 9}~~32, 35, 38 or 40~~, wherein the host cell is selected from the group consisting of a bacteria, yeast, insect or mammalian cell.

¹²~~43~~. (New) The method of any one of claims ^{1 4 7 9}~~32, 35, 38 or 40~~, wherein the host cell is a bacteria cell.

¹³~~44~~. (New) The method of any one of claims ^{1 4 7 9}~~32, 35, 38 or 40~~, wherein the host cell is *E. coli*.

¹⁴~~45~~. (New) The method of any one of claims ^{1 4 7 9}~~32, 35, 38 or 40~~, wherein the host cell is a mammalian cell.

¹⁵~~46~~. (New) The method of any one of claims ^{1 4 7 9}~~32, 35, 38 or 40~~, wherein the soluble protein is a recombinant protein.

¹⁵~~47~~. (New) The method of claim ¹⁵~~46~~, wherein the recombinant protein is a cysteine mutein of a member of the growth hormone supergene family.

¹⁷~~48~~. (New) The method of claim ¹⁵~~47~~, wherein the member of the growth hormone supergene family is selected from the group consisting of: growth hormone, erythropoietin and alpha interferon.

¹⁸~~49~~. (New) The method of claim ¹⁵~~46~~, wherein the recombinant protein is a cysteine mutein of a protein selected from the group consisting of a member of the TGF-beta superfamily, platelet derived growth factor-A, platelet derived growth factor-B, nerve growth factor, brain derived neurotrophic factor, neurotrophin-3, neurotrophin-4, vascular endothelial growth factor, endostatin, angiostatin.

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20. (New) The method of claim ~~46~~¹⁵, wherein the recombinant protein is a cysteine munein of a heavy or light chain of an immunoglobulin. 1 4 7 9
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21. (New) The method of any one of claims ~~32, 35, 38~~ or ~~40~~, wherein the cysteine blocking agent is a thiol-reactive compound. 1 4 7 9
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22. (New) The method of any one of claims ~~32~~ or ~~38~~, wherein the cysteine blocking agent is selected from the group consisting of cystine, ~~cystamine~~, dithioglycolic acid, or oxidized glutathione, or a derivative thereof. ~~or a derivative thereof~~ ✓ 1 4 7 9
- 22
23. (New) The method of any one of claims ~~32, 35, 38~~ or ~~40~~, wherein the cysteine blocking agent is cystine. 1 4 7 9
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24. (New) The method of any one of claims ~~32, 35, 38~~ or ~~40~~, wherein the concentration of the cysteine blocking agent is in the range of about 0.1 micromolar to 100 millimolar. 1 4 7 9
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25. (New) The method of any one of claims ~~32, 35, 38~~ or ~~40~~, wherein the concentration of the cysteine blocking agent is in the range of about 50 micromolar to 5 millimolar. 1 4 7 9
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26. (New) The method of any one of claims ~~32, 35, 38~~ or ~~40~~, further comprising the steps of:
- d) reducing the isolated protein with a disulfide reducing agent; and
 - e) exposing the protein to a cysteine-reactive moiety to obtain a cysteine-modified protein.
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27. (New) The method of claim ~~56~~²⁵, further comprising isolating the cysteine-modified protein from the unmodified protein. ✓
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28. (New) The method of claim ~~56~~, wherein the cysteine-reactive moiety is a polyethylene glycol.
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29. (New) The method of claim ~~56~~²⁵, wherein the isolated protein is a cysteine munein of growth hormone. ✓
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30. (New) The method of claim ~~56~~²⁵, wherein the isolated protein is a cysteine munein of erythropoietin.

³⁰~~61~~. (New) The method of claim 56, wherein the isolated protein is a cysteine murein of alpha interferon-2.

³¹~~62~~. (New) A PEGylated growth hormone protein comprising at least one free cysteine and having an EC₅₀ of less than about 400 ng/ml in an in vitro bioassay, obtained by the methods of any one of claims 32, 35, 38 or 40.

³¹~~32~~~~63~~. (New) The PEGylated growth hormone protein of claim ~~62~~, wherein a polyethylene glycol is attached to at least one amino acid located in the region preceding helix A of growth hormone.

³¹~~33~~~~64~~. (New) The PEGylated growth hormone protein of claim ~~62~~, wherein a polyethylene glycol is attached to the amino acid at position 3 of growth hormone.

³¹~~34~~~~65~~. (New) The PEGylated growth hormone protein of claim ~~62~~, wherein a polyethylene glycol is attached to a cysteine residue substituted for threonine-3 of growth hormone.

³¹~~35~~~~66~~. (New) The PEGylated growth hormone protein of claim ~~62~~, wherein a polyethylene glycol is attached to at least one amino acid located in the C-D loop of growth hormone.

³¹~~36~~~~67~~. (New) The PEGylated growth hormone protein of claim ~~62~~, wherein a polyethylene glycol is attached to the amino acid at position 144 of growth hormone.

³¹~~37~~~~68~~. (New) The PEGylated growth hormone protein of claim ~~62~~, wherein a polyethylene glycol is attached to a cysteine residue substituted for serine-144 of growth hormone.

³⁸~~38~~~~69~~. (New) A PEGylated erythropoietin protein comprising at least one free cysteine and having an EC₅₀ of less than about 1000 ng/ml in an in vitro bioassay, obtained by the methods of any one of claims 32, 35, 38 or 40.

³⁸~~39~~~~70~~. (New) The PEGylated erythropoietin protein of claim ~~69~~, wherein a polyethylene glycol is attached to at least one amino acid located in the A-B loop of erythropoietin.

³⁸~~40~~~~71~~. (New) The PEGylated erythropoietin protein of claim ~~69~~, wherein a polyethylene glycol is attached to the amino acid at position 26 of erythropoietin.

~~41~~ 72. (New) The PEGylated erythropoietin protein of claim ~~69~~³⁸, wherein a polyethylene glycol is attached to a cysteine residue substituted for threonine-26 of erythropoietin.

~~42~~ 73. (New) The PEGylated erythropoietin protein of claim ~~69~~³⁸, wherein a polyethylene glycol is attached to at least one amino acid located in the C-D loop of erythropoietin.

~~43~~ 74. (New) The PEGylated erythropoietin protein of claim ~~69~~³⁸, wherein a polyethylene glycol is attached to the amino acid at position 126 of erythropoietin.

~~44~~ 75. (New) The PEGylated erythropoietin protein of claim ~~69~~³⁸, wherein a polyethylene glycol is attached to a cysteine residue substituted for serine-126 of erythropoietin.

~~45~~ 76. (New) A PEGylated alpha interferon protein comprising at least one free cysteine and having an IC_{50} of less than about 1900 pg/ml in an in vitro bioassay, obtained by the methods of any one of claims 32, 35, 38 or 40.

~~46~~ 77. (New) The PEGylated alpha interferon protein of claim ~~76~~⁴⁵, wherein the alpha interferon protein is selected from the group consisting of a naturally occurring alpha interferon, a non-natural alpha interferon comprising parts of two or more naturally occurring alpha interferons, or a consensus alpha interferon.

~~47~~ 78. (New) The PEGylated alpha interferon protein of claim ~~76~~⁴⁵, wherein the alpha interferon protein is alpha interferon-2.

~~48~~ 79. (New) The PEGylated alpha interferon protein of claim ~~78~~⁴⁸, wherein a polyethylene glycol is attached to at least one amino acid located in the region preceding helix A of alpha interferon-2.

~~49~~ 80. (New) The PEGylated alpha interferon protein of claim ~~78~~⁴⁸, wherein a polyethylene glycol is attached to the amino acid at position 5 of alpha interferon-2.

~~50~~ 81. (New) The PEGylated alpha interferon protein of claim ~~78~~⁴⁸, wherein a polyethylene glycol is attached to a cysteine residue substituted for glutamine-5 of alpha interferon-2.

~~51~~ 82. (New) The PEGylated alpha interferon protein of claim ~~78~~⁴⁸, wherein a polyethylene glycol is attached to at least one amino acid located in the region following helix E of alpha interferon-2.

~~52~~ 83. (New) The PEGylated alpha interferon protein of claim ~~78~~⁴⁸, wherein a polyethylene glycol is attached to the amino acid at position 163 of alpha interferon-2.

~~53~~ 84. (New) The PEGylated alpha interferon protein of claim ~~78~~⁴⁸, wherein a polyethylene glycol is attached to a cysteine residue substituted for serine-163 of alpha interferon-2.

~~54~~ 85. (New) The PEGylated alpha interferon protein of claim ~~78~~⁴⁸, wherein a polyethylene glycol is attached to at least one amino acid located in the C-D loop of alpha interferon-2.